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Suppression of Type 1 Insulin-like Growth Factor Receptor Expression by Small Interfering RNA Inhibits A549 Human Lung Cancer Cell Invasion *in vitro* and Metastasis in Xenograft Nude Mice

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Abstract Cancer invasion and metastasis, involving a variety of pathological processes and cytophysiological changes, contribute to the high mortality of lung cancer. The type 1 insulin-like growth factor receptor (IGF-1R), associated with cancer progression and invasion, is a potential anti-invasion and anti-metastasis target in lung cancer. To inhibit the invasive properties of lung cancer cells, we successfully down-regulated IGF-1R gene expression in A549 human lung cancer cells by small interfering RNA (siRNA) technology, and evaluated its effects on invasion-related gene expression, tumor cell *in vitro* invasion, and metastasis in xenograft nude mice. A549 cells transfected with a plasmid expressing hairpin siRNA for IGF-1R showed a significantly decreased IGF-1R expression at the mRNA level as well as the protein level. In biological assays, transfected A549 cells showed a significant reduction of cell-matrix adhesion, migration and invasion. Consistent with these results, we found that down-regulation of IGR-1R concomitantly accompanied by a large reduction in invasion-related gene expressions, including MMP-2, MMP-9, u-PA, and IGF-1R specific downstream p-Akt. Direct tail vein injections of plasmid expressing hairpin siRNA for IGF-1R significantly inhibited the formation of lung metastases in nude mice. Our results showed the therapeutic potential of siRNA as a method for gene therapy in inhibiting lung cancer invasion and metastasis.

Key words lung cancer; invasion; metastasis; type 1 insulin-like growth factor receptor; small interfering RNA

Lung cancer is the leading cause of cancer death, accounting for 19% of all cancer deaths worldwide. That percentage is increasing by 0.5% each year. Non-small cell lung cancer accounts for approximately 80% of all lung cancers, and has a low five-year survival rate (8%–14%) [1]. This high mortality is due largely to tumor invasion and metastasis [2–4], especially early invasion and metastasis [5]. Thus, inhibition of metastasis is a potential therapeutic strategy in treating lung cancer [6]. Despite the advances in chemotherapy and radiation therapy, the current treatments for tumor invasion and metastasis are still far from satisfaction. Therefore, novel strategies in the treatment of lung cancer are urgently needed [7,8].

The type 1 insulin-like growth factor receptor (IGF-1R) (GenBank accession No. NM_000875), recognized as one of the most important targets in anti-proliferation, is a potential anti-invasion and antimetastasis target in lung cancer [9,10]. Studies have shown that IGF-1R, which acts in an autocrine/paracrine manner, is overexpressed in different lung cancer cells [11–13], leading to an enhancement of invasion and metastasis [9,10,14–17]. Furthermore, studies have also shown that inhibition of

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IGF-1R by various strategies, such as antisense oligonucleotides [15,18] and introduction of the inactive form [19–21] of the IGF-1R, could inhibit cancer cell invasion *in vitro* as well as metastasis in xenograft nude mice. However, there are problems with regard to delivery, stability and off-target effects [22]. To deal with these problems, the small interfering RNA (siRNA) strategy has recently been developed. siRNA can inhibit the expression of particular proteins more efficiently and specifically compared with the antisense approach [22,23] and has been adopted as a promising technology in gene therapy.

In the present study, we aimed to knockdown IGF-1R expression in lung cancer cells using siRNA, then evaluated its effects on invasion-related genes in the IGF-1R-mediated signaling pathway, and on *in vitro* invasion and metastasis of lung cancer cells in xenograft nude mice.

Materials and Methods

Materials

IGF-1R McAb (MS-645-P0) was from Lab Vision (New York, USA). Matrix metalloproteinase (MMP)-2 PcAb (sc-13595), MMP-9 McAb (sc-6840), human β-actin PcAb (sc-1616), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (sc-2060) and HRP-conjugated goat anti-rabbit IgG (sc-2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Akt McAb (2966) and p-Akt McAb (4056) were from Cell Signaling Technology (Beverly, USA). Urokinase plasminogen activator (u-PA) McAb was from Sigma (St. Louis, USA).

Construction of plasmid expressing siRNA for IGF-1R

IGF-1R-specific siRNA was designed with BLOCK-iT RNAi designer (http://www.rnaidesigner.invitrogen.com), and synthesized by Sangon (Shanghai, China). Two pairs of IGF-1R-specific siRNA (siIGF-1R1 and siIGF-1R2) were selected, and an unrelated siRNA (si-control) was used as the negative control. Plasmids (psiIGF-1R1, psiIGF-1R2 and psi-control) expressing siRNA were under the control of the human U6 promoter in pENTR/U6 vector (BLOCK-iT U6 RNAi entry vector kit; Invitrogen, Carlsbad, USA), and the complimentary sequences were as follows: pENTRTM/U6-IGF-1R-siRNA1, 5′-caccgcacaattactgctccaaagacgaatctttggagcagtaattgtgc-3′; pENTRTM/U6-IGF-1R-siRNA2, 5′-caccgccgatgtgtgagaagaccttcaagagaggtcttctcacacatcggc-3′; pENTRTM/U6-unrelated-siRNA, 5′-caccgctcaccggctccagatttatcgaaataaatctggagccggtgagc-3′.

Cell culture and transfection

A highly metastatic A549 human lung carcinoma cell line, which belongs to non-small cell lung cancer with overexpression of IGF-1R, was a gift from Dr. Xiaoyong LU (First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China). A549 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37 ºC. Cells were subcultured every 3 d. Two milliliters of A549 cell suspension was seeded in a 6-well plate at the concentration of 3×10^5 cells/ml and incubated for 16 h. Cells were then transfected with siRNA plasmid (IGF-1R-specific siRNA expressing plasmid) or control plasmid (negative control siRNA expressing plasmid) using Lipofectamine (Invitrogen) according to the manufacturer's protocols. Cells (A549-siIGF-1R1, A549-siIGF-1R2 and A549-control) were incubated in 2 ml of serum-free medium (SFM) per well for 6 h, and then 1 ml RPMI medium (with or without 10% FBS) was added. All assays were performed 48 h post-transfection. The transfected cells for Western blot analysis of Akt were incubated in SFM for 6 h, in RPMI 1640 medium with 10% FBS for 24 h, and again in SFM for 16 h prior to a 30 min serum stimulation. The transfected cells for MMP and u-PA activity detection by zymography were incubated in SFM for 24 h.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

After transfection, total RNA was extracted using Trizol reagent (Invitrogen) from cells according to the manufacturer's instructions, and stored at –80 ºC until use. The first strand cDNA was generated from 4 μg of total RNA in a final volume of 20 μl using Moloney murine leukemia virus RT (Promega, Madison, USA). PCR amplification was performed using a PTC-200 DNA thermal cycler (Perkin-Elmer, Foster City, USA). The sequences of PCR primers were: IGF-1R, 5′-aaatgtgcccgagcgtgtg-3′ (forward) and 5′-tgcccttgaagatggtgcatc-3′ (reverse); MMP-2, 5′-cactttcctgggcaacaaat-3′ (forward) and 5′-tgatgtcatcctgggacaga-3′ (reverse); MMP-9, 5′-ctggcttagatcattcctca-3′ (forward) and 5′-agtacttcccatccttgaacaaata-3′ (reverse). The primers of u-PA were designed as described previously [24], and the sequences were as follows: 5′-agaattcaccaccatcgaga-3′ (forward) and 5′-atcagcttcacaacagtcat-3′ (reverse). The primers for β-actin were from another previous report [25], and the sequences were as follows: 5′-gacaggatgcagaaggagat-3′

(forward) and 5′-tgcttgctgatccacatctg-3′ (reverse). IGF-1R and MMP-2 samples were subjected to 35 PCR cycles. MMP-9, u-PA and β-actin samples were subjected to 30, 40 and 25 PCR cycles, respectively. Each thermocycling was performed according to the following profile: 94 ºC for 3 min; denaturation for 30 min at 94 ºC, annealing for 53.5 ºC for 30 s for IGF-1R and β-actin, or 1 min at 56 ºC for u-PA, or 50 s at 55 ºC for MMP-2, or 30 s at 55 ºC for MMP-9, and extensing for 1 min at 72 ºC (30 s for MMP-9); and a final extension at 72 ºC for 10 min. PCR product (10 μl) was analyzed by 2% agarose gel electrophoresis. Experiments were performed at least three times.

Western blot analysis of IGF-1R, MMP-9, MMP-2 and u-PA

For analysis of MMP-2, MMP-9 and u-PA, 50-fold concentrated conditional medium from treated cells was used. To prepare the medium, the transfected cells were incubated in SFM for 48 h. At the end of incubation, the conditional medium was harvested, centrifuged to remove cellular debris and concentrated at −80 ºC using Refrigeration and vacuum system plus with vapornet UVS400A (Savant Instruments, Hicksville, USA). For analysis of IGF-1R and human β-actin, cell extracts were used. These were prepared by incubation in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1.2% Triton-X114, 1 mM NaF, 200 μM $NaVO_4$) containing 1 protease inhibiter cocktail tablet (Roche, Basel, Switzerland) per 10 ml of buffer for 10 min at 4 ºC. Protein concentrations were determined using a BCA protein assay reagent kit (Pierce Biotechnology, Rockford, USA). Cell extracts (or concentrated conditional medium) containing 100 μg of total proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham, Piscataway, USA). The membranes were subsequently blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), or phosphate-buffered saline containing 1% Tween-20 (PBS-T) without skimmed milk for IGF-1R, for 0.5 h, incubated with a primary antibody (1:200 dilution for IGF-1R, MMP-2, MMP-9 and u-PA; 1:1000 dilution for human β-actin) overnight at 4 ºC, or 2 h at room temperature for human β-actin, then incubated with HRP-conjugated goat anti-mouse (or goat anti-rabbit) IgG (1:2500 dilution) for 1 h at room temperature. Intensive TBS-T (PBS-T for IGF-1R) washing was performed between two incubations. The blots were developed according to chemiluminescence protocol (No. 12015200001; Roche), and analyzed using Scion image software (National Institutes of Health, Maryland, USA). Human β-actin was used as an internal control. Experiments were performed in triplicate.

Western blot analysis of phospho-Akt

The transfected A549 cells were subsequently incubated in SFM for 6 h, or in RPMI 1640 medium with 10% FBS for 24 h, and again in SFM for 16 h prior to serum stimulation for 30 min, and the total cellular proteins were extracted by RIPA and a protease inhibiter cocktail according to the methods reported previously [26]. Cell extracts containing 100 μg of total cellular proteins were resolved by 10% SDS-PAGE and probed with antibodies to Akt and phospho-Akt (1:200 dilution). HRP-conjugated goat anti-mouse or goat anti-rabbit IgG antibody (1:2500 dilution) was used as the secondary antibody. Experiments were performed at least three times.

Analysis of MMP-2, MMP-9 and u-PA activities by zymography

The activities of MMP-2 and MMP-9 of the conditional medium were measured by gelatin-zymogram protease assay as previously described [27] with some modifications. Briefly, treated A549 cells of equal number (1×10^5 cells per 24-well culture plate) were left untreated in SFM at 37 ºC with 5% CO_2 for 24 h. Cell culture supernatants were collected, clarified by centrifugation, and mixed with substrate gel sample buffer without β-mercaptoethanol [40% (*V*/*V*) glycerol, 0.25 M Tris-HCl, pH 6.8, and 0.1% bromophenol blue] at a ratio of 3:1. The prepared samples (60 μg) without being boiled were subjected to electrophoresis at 4 ºC with 10% SDS-PAGE containing 0.1% gelatin (Sigma). After electrophoresis, the gels were washed twice using 2.5% Triton-X 100 for 30 min, incubated with reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, and 0.02% NaN_3) for 18 h at 37 ºC, stained with 0.05% Coomassie blue G-250, then destained in 10% acetic acid and 20% methanol. Gelatinolytic activity was revealed as clear bands against a blue-stained background. The experiment was carried out three times in succession.

Visualization of u-PA activity was performed by casein-plasminogen zymography as previously described [27]. Briefly, 2% (*W*/*V*) casein (Sigma) and 20 mg/ml plasminogen (Sigma) were added to 8% SDS-PAGE gel. Samples with a total protein of approximately 20 μg were then loaded onto the gels. The u-PA activity was measured as described in the gelatin zymography. Experiments were performed in triplicate.

Cell-matrix adhesion assay

The cell-matrix adhesion assay was carried out in 96-well plates precoated with 30 μg/well Matrigel which acted as the artificial extracellular matrix (ECM), and left to air-dry for 40 min according to the method of Zhang *et al*. [24] with some modifications. Briefly, the treated cells were harvested and resuspended in RPMI 1640, 2×10^5 cells in 200 μl of medium were added into each well. After incubation for 2 h, nonadherent cells were carefully removed by washing with warm PBS. The adherent cells were fixed in 100% methanol and counted under a microscope (Olympus, Tokyo, Japan). Experiments were performed in triplicate.

Cell invasion assay

In vitro invasion assay was performed in a transwell system (Corning, Corning, USA) as previously reported [27] with some modifications. Briefly, Matrigel (3.9 mg/ml, 60–80 ml) was added to the upper surface of a polycarbonic membrane (diameter 6.5 mm, pore size 8 mm) to form a thin gel layer, which served as the ECM. The filter was dried in a laminar hood overnight, and then reconstituted with 100 μl of PBS at 37 ºC for 2 h. The upper compartment of the filter contained the treated cells at a density of 1×10^5 cells/well in 50 μl of SFM. The bottom filter was filled with 200 μl of conditional medium derived from NIH3T3 cells. After 24 h incubation at 37 ºC with 5% CO_2, the polycarbonic membrane was fixed with 100% methanol for 10 min and stained with 0.2% crystal violet solution, then the cells on the upper surface were completely removed by wiping with a cotton swab. Cells that had penetrated to the lower surface of the filter were counted under an Olympus microscope in three randomized fields at a magnification of 200×.

Cell migration assay

Cell migration assay was carried out in a transwell filter on membrane filters not coated with Matrigel. Migration of cells treated with siRNA-IGF-1R or si-control was measured as described in the invasion assay as previously described [27]. Each assay was performed at least three times.

Cell metastasis in xenograft nude mice

For the experimental metastasis studies, nude male mice (4–5 weeks old) received a tail vein injection of 0.1 ml of A549 cancer cell suspension containing 2×10^6 cells transiently transfected with psiIGF-1R or psi-control 48 h before the animal injection as previously described [25] with some modifications. Because synthetic siRNA-mediated RNA interfering in human cells is transitory, with cells recovering from a single treatment in 4–6 d, the mice were treated with psiIGF-1R twice per week (0.5 μg/g body weight by tail vein injection) after injection of the tumor cells [25]. Mice were killed by cervical dislocation on day 20, and heart and lung were removed quickly at 4 ºC. The right lungs were analyzed by counting the number of surface nodules as indices of metastatic burden. The left lungs were snap-frozen in liquid nitrogen and subjected to hematoxylin-eosin histostaining for the evaluation of the presence or absence of tumors, and RT-PCR analysis for detecting the mRNA expression of the human housekeeping gene, hypoxanthine-guanine-phosphoribosyltransferase (hHPRT), and IGF-1R gene. The PCR primers of hHPRT were designed according to a previous report [25], and the sequences were as follows: 5′-ttccttggtcaggcagtataatcc-3′ (forward) and 5′-agtctggcttatatccaacacttcg-3′ (reverse). These experiments were repeated twice with five mice per condition for each experiment.

Statistical analysis

All quantitative data were presented as mean±SD. A commercially available software package, SPSS version 10.0 (SPSS, Chicago, USA), was used for statistical analysis. Student's two-tailed *t*-test in two groups and one-way ANOVA in multiple groups were used to analyze the statistical significance of differences. The differences among groups were interpreted as statistically significant if the *P* value was less than 0.05.

Results

siIGF-1R significantly down-regulates IGF-1R expression at both mRNA and protein levels in lung cancer cell line A549

To efficiently knock down the IGF-1R expression in invasive A549 cells, two siRNA expression plasmids were prepared. We detected the level of mRNA expression in the transfectants using RT-PCR after 48 h transfection. The data showed that the level of IGF-1R mRNA was significantly decreased in siRNA1-A549 cells [(20.1±3.4)% of that in si-control group] compared with si-control cells, the inhibition rate of siIGF-1R1 for IGF-1R mRNA expression was up to 79.9% of the si-control [**Fig. 1(A)**]. Furthermore, the result of RT-PCR was confirmed by Western blot analysis using IGF-1R-specific McAb. The

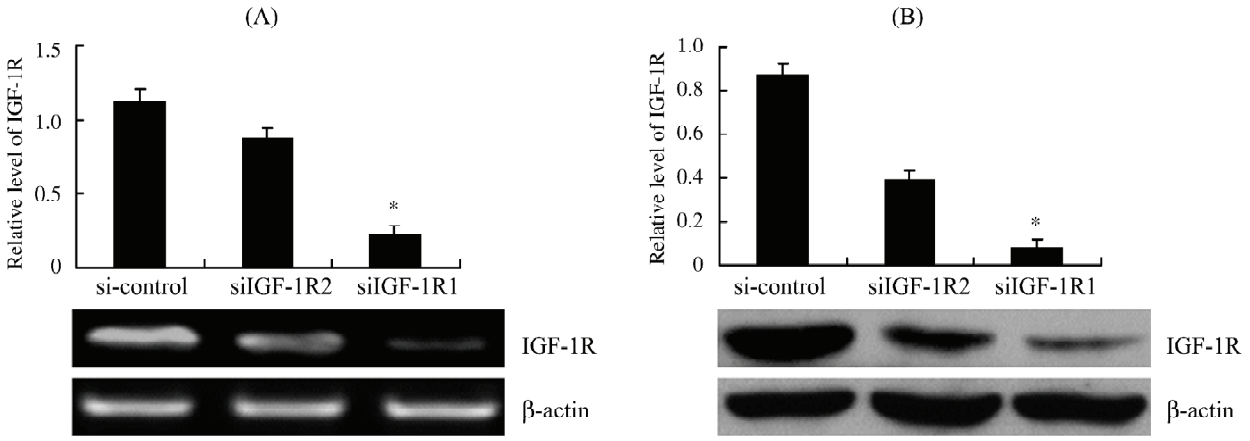

Fig. 1 Small interfering RNA (siRNA) inhibits type 1 insulin-like growth factor receptor (IGF-1R) expression in A549 human lung cancer cells

(A) After 48 h of treatment with siIGF-1R or si-control, 4 μg total RNA was used for reverse transcription–polymerase chain reaction analysis of IGF-1R mRNA. (B) Western blot analysis was conducted on cell lysates with antibodies to IGF-1R. β-actin was used as an internal control. *P<0.05 versus negative control vector.

level of protein expression of IGF-1R in A549-siIGF-1R1 cells was decreased by (90.8±4.9)% [**Fig. 1(B)**]. Similar but less profound knockdown of IGF-1R expression at both mRNA and protein levels was induced by siIGF-1R2 (**Fig. 1**). These results indicated that highly significant and specific down-regulation of IGF-1R expression with siIGF-1R1 is available by careful selection of the targeted sequence.

Down-regulation of IGF-1R decreases MMP-2, MMP-9 and u-PA expression levels and decreases Akt kinase activity in A549 cells

Previous results have indicated that constitutive activation of IGF-1R and ligands induces the expression and activation of invasion-related genes including MMP-2, MMP-9 and u-PA [28]. We further explored the effects of IGF-1R silence on these invasion-related genes. The results showed that MMP-2, MMP-9 and u-PA expression at mRNA [**Fig. 2(A)**] and protein [**Fig. 2(A)**] levels were significantly decreased in A549-siIGF-1R1 cells compared with those in si-control cells. Furthermore, as shown in **Fig. 2(C)**, the activities of MMP-2, MMP-9 and u-PA, as assayed by gelatinase zymography and casein-plasminogen zymography, were significantly reduced in the supernatant of A549-siIGF-1R1 cells compared with the negative control. Similar but less dramatic results were obtained for the expression levels of MMP-2, MMP-9 and u-PA in the supernatant from A549-siIGF-1R2 cultured cells compared with the negative control. The results suggested that siIGF-1R could greatly decrease invasion-related gene expression.

Studies have shown that Akt is one of the predominant downstream targets of IGF-1R associated with cellular invasion [9,17,29,30]. To better understand the effect of siRNA-mediated down-regulation of IGF-1R on signaling pathways, the total and phospho-Akt levels were assayed. As shown in **Fig. 2(D)**, phospho-Akt level, as detected by Western blot analysis, displayed a significant reduction in A549-siIGF-1R1 compared with the control after IGF-1R knockdown. However, the total Akt level was not markedly influenced in A549 cells. The results suggested that siIGF-1R could greatly decrease Akt kinase activation.

siIGF-1R inhibits cell-matrix adhesion, migration and invasion of A549 cells

To evaluate the effect of siIGF-1R on the adhesion of tumor cells to ECM, tumor cell-matrix adhesion assay was performed. The number of attached cells in the siIGF-1R group was compared with the control. siIGF-1R1 significantly inhibited the adhesion of A549 cells to Matrigel by (52.7±3.5)% compared with the negative control [**Fig. 3(A)**]. The inhibitory effect of siIGF-1R on invasion in A549 cells was further confirmed by the Matrigel invasion assay, the determined ability of cells to invade a matrix barrier containing Matrigel, and the migration assay, the ability of cells to migrate through polycarbonic membrane not containing Matrigel. As illustrated in **Fig. 3(B,C)**, A549-siIGF-1R cells showed a low level of penetration through the Matrigel-coated or uncoated membranes compared with the A549-control cells. Quantification results

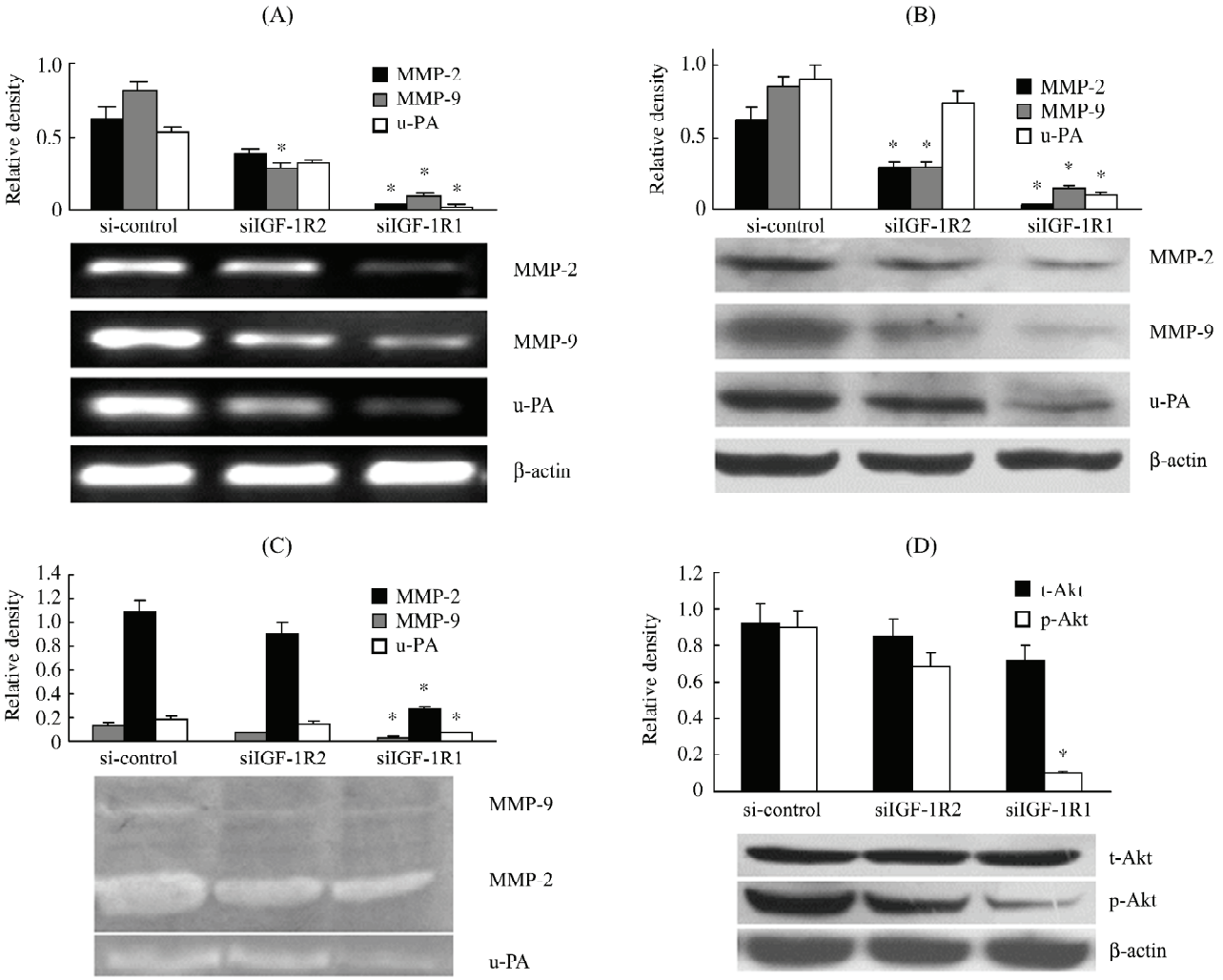

Fig. 2 Down-regulation of type 1 insulin-like growth factor receptor (IGF-1R) decreases matrix metalloproteinase (MMP)-2, MMP-9 and urokinase plasminogen activator (u-PA) expression levels, and decreased phosphorylation of Akt in A549 human lung cancer cells

A549 cells were transfected with psiIGF-1R or psi-control for 48 h. (A) Total RNA was isolated, and reverse transcription-polymerase chain reaction analysis was carried out as described in "Materials and Methods". (B) Equal amounts of conditional medium were applied to each lane, and immunoblot analysis was carried out using the anti-MMP-2, anti-MMP-9 and anti-u-PA antibody. (C) Activities of MMP-2, MMP-9 and u-PA in conditional medium of the cell culture was evaluated by gelatin zymography and casein zymography. (D) Following the indicated treatment as described in "Materials and Methods", total cellular proteins were extracted and analyzed by Western blotting using total Akt (t-Akt) or phospho-Akt (p-Akt) McAb. β-actin was used as an internal control. *P<0.05 versus negative control vector.

confirmed that A549-siIGF-1R1 cells significantly reduced migration and invasion by (66.1±7.6)% and (64.4±5.4)%, respectively, when compared with that of negative control, respectively. Moreover, the inhibitory effects of siIGF-1R2 on cell motility and invasion were observed, with only (38.4±3.9)% and (50.6±5.7)% remaining, respectively, after siIGF-1R2 treatment [**Fig. 3(B,C)**]. These results were consistent with the studies above showing that siIGF-1R2 was less effective in the knock-down of the expression of IGF-1R and in decreasing the expression of invasion-related genes in these cells than siIGF-1R1.

Transfection of psiIGF-1R inhibits A549 cell lung colonization

To further elucidate the effect of IGF-1R silence on the inhibition of metastatic potential, a lung metastasis model was established using mice. The metastatic potential of

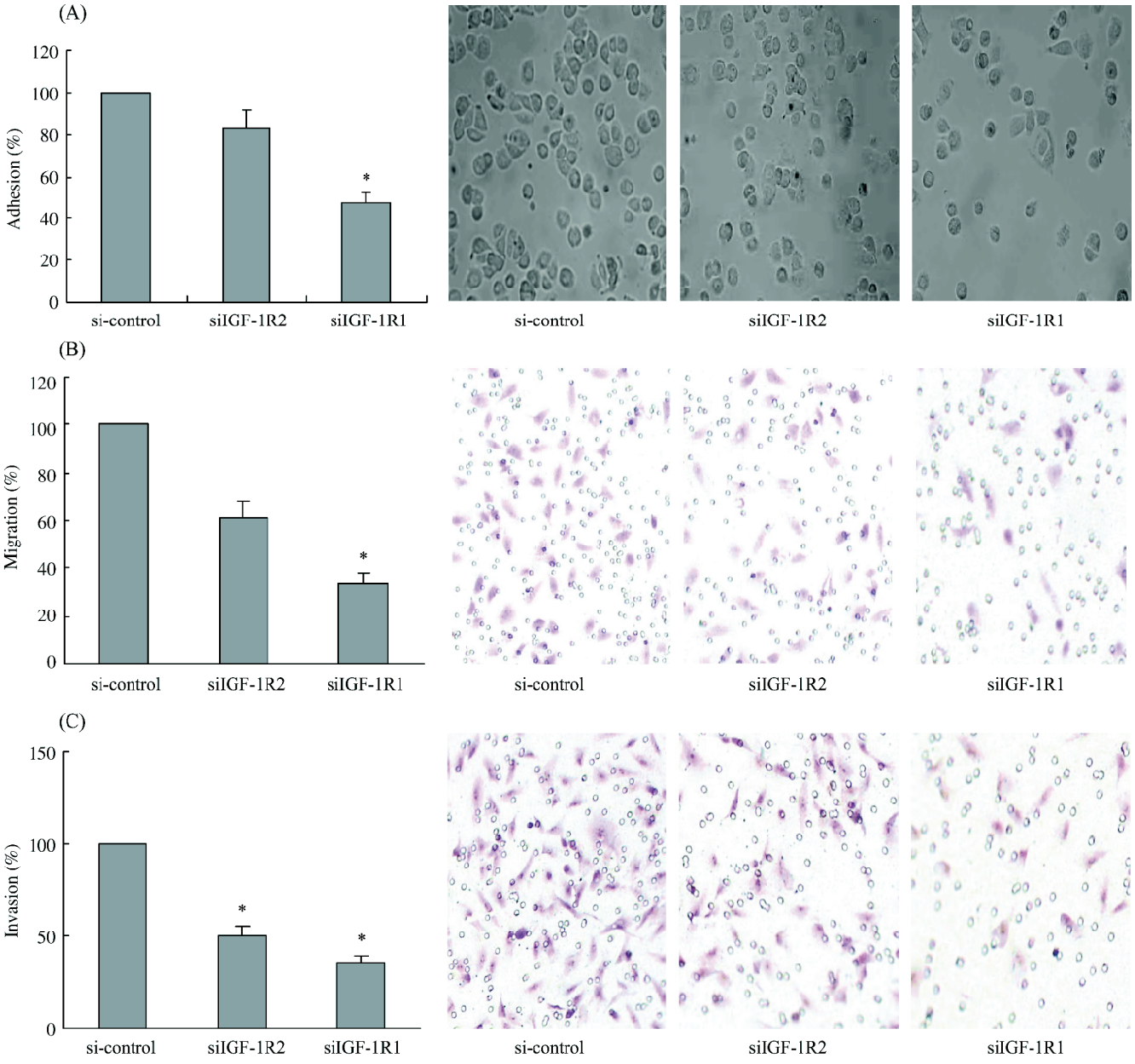

Fig. 3 Impact of type 1 insulin-like growth factor receptor (IGF-1R) silencing on A549 human lung cancer cell adhesion, migration and invasion

After transfection and incubation for 48 h, A549 cells were subjected to analysis for cell-matrix adhesion (A), motility (B) and invasion (C) as described in "Materials and Methods". Determined inhibition of siIGF-1R was quantified, with that of the control being 100%. **P*<0.05 versus negative control vector. Magnification, 200×.

A549 cells treated with siIGF-1R or si-control was determined by the formation of micrometastases and the mRNA expression of hHPRT that does not cross-react with its mouse counterpart in mouse lung. The metastatic foci, which were seen on the lung surface and under a dissecting microscope, occurred clearly in lungs of animals treated with psiIGF-1R2 or psi-control [**Fig. 4 (A,B)**]. **Fig. 4(B)** shows that the cancer cells varied in size and shape (circular or irregular), and the nuclei also differed in size and displayed pathologic mitosis. Quantification assessment showed that the presence of metastatic foci was significantly decreased in the siIGF-1R1 group compared with the control group [**Fig. 4(C,D)**]. As shown in **Fig. 4(E)**, these results were confirmed by the

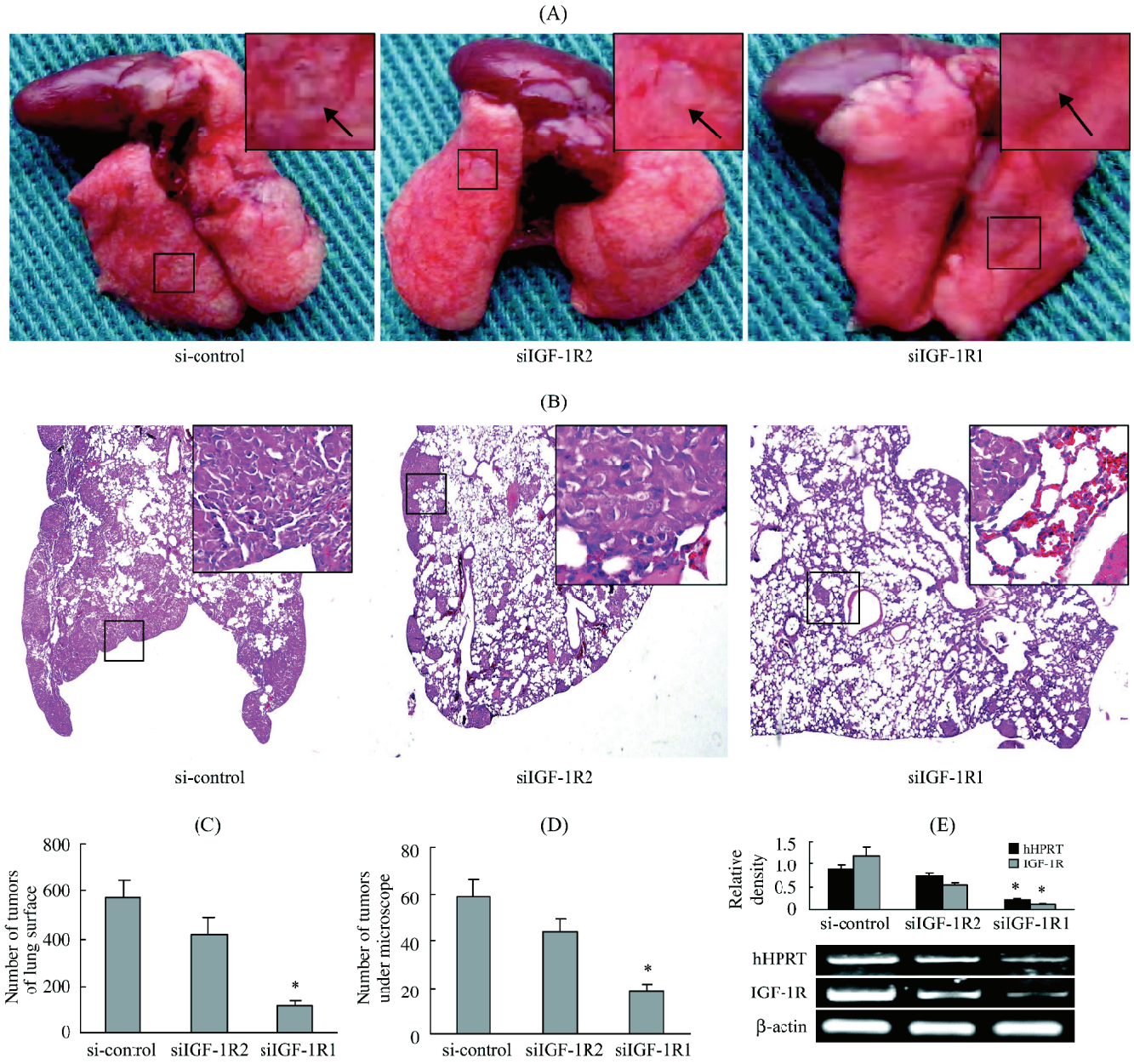

Fig. 4 Inhibition of type 1 insulin-like growth factor receptor (IGF-1R) inhibits human lung cancer metastasis in nude mouse model

Nude mice received tail vein injection of transfected A549 cancer cell suspension and treated with siIGF-1R or si-control twice a week. Twenty days later, the lung was harvested. (A,B) The metastatic foci are shown on the lung surface and under a dissecting microscope at original magnification (4×). Selected areas are shown in the upper right-hand corners at larger magnification (80×). (C,D) Quantification assessment of the number of metastatic foci showed the incidence of metastatic foci in lungs of mice. (E) Reverse transcription-polymerase chain reaction was used to analyze the mRNA expression of hHPRT and IGF-1R genes. β-actin was used as an internal control. *P<0.05 versus negative control vector.

slight expression of hHPRT in the siIGF-1R1 group. There was a decreased trend in the siIGF-1R2 group compared with the control group, but without significant differences in statistics. Variation of the expression of the human-specific IGF-1R gene in metastasis-infiltrated lungs in each group was in line with the results of **Fig. 1(A)**. These results indicate that siIGF-1R has the potential to suppress A549 cell lung colonization.

Discussion

IGF-1R, a type II receptor tyrosine kinase, consists of two α-chains (130–135 kDa) and two β-chains (90–95 kDa) and is formed as a heterotetramer ($\alpha_2\beta_2$) [10]. The β-subunits have highly conserved tyrosine kinase domains with several tyrosines in the C-terminal domain at position 1250, 1251, and 1316, some of which are known to regulate cell migration, invasion, and metastasis [18]. Activation of the intrinsic IGF-1R tyrosine kinase results in activation of specific downstream, including the major control elements, phosphatidylinositol 3′-kinase (PI3-K)/Akt and MAPK. PI3-K/Akt plays an important role in cancer invasion and metastasis [17,31]. There is growing evidence from both laboratory and population studies showing that over-expressed IGF-1R is related to tumor malignant type, promoting cell proliferation, invasion and metastasis [32]. On the basis of suppressing the IGF-1R signal pathway, several approaches to inhibit tumor invasion and metastasis have been reported in some cancer cell lines including lung cancer cell line, such as using antisense oligonucleotides [15,18], and the introduction of the inactive form of IGF-1R [19–21]. However, the stability, delivery efficacy and off-target effects of these agents seem to be crucial limiting factors in exerting an inhibitory effect on the targeted molecules. Due to superior potency, stability, specificity, versatility and ease in identifying effective target sites, siRNA was demonstrated to have promising potential in gene therapy [22,23]. Therefore, we developed two plasmid vectors expressing small hairpin RNAs targeted at IGF-1R, siIGF-1R1 and siIGF-1R2, which significantly inhibit the invasive potential of the cancer cell in this study. It was found that siRNA could effectively silence IGF-1R gene expression at the mRNA and protein levels in A549 cells. Different siRNAs always result in different degrees of efficiency in silencing IGF-1R [33,34], and our data showed that siIGF-1R1 achieved even more suppression of IGF-1R expressions than siIGF-1R2.

Current theory holds that tumor metastasis is a dynamic process involving many components, including tumor cell adhesion, ECM proteolysis, migration, invasion and colonization. Disruption of any one of these steps could potentially lead to abrogation of the entire process. The impacts of suppression of IGF-1R on ECM degradation were investigated in this study. Usually, ECM, mainly being composed of laminin and type IV collagen, constitutes the first barrier against tumor spread. It has been demonstrated that cancer cell adhesion to ECM, which is an early step to the destruction of ECM, depends on the presence of membrane-spanning integrins [35]. Previous studies confirmed that IGF-1R and its ligands might affect the expression of integrins and activate the specific integrins [36–38]. Therefore, the decreased expression of IGF-1R could reduce adhesion to ECM [36]. Our results showed that knockdown of IGF-1R, using siIGF-1R1, significantly decreases A549 cell adhesion to the ECM. Another crucial step of destruction of ECM requires activation of proteolytic enzymes, such as MMP-2, MMP-9 and u-PA [28]. u-PA converts the zymogen plasminogen to plasmin, which prompts tumor invasion by directly degrading ECM components and activating latent collagenases and metalloproteases [39]. The key enzymes of the MMP family, 72 kDa type IV collagenase (MMP-2) and 92 kDa type IV collagenase (MMP-9), degrade type IV collagen to increase cancer invasion and metastasis [40]. Recent studies have provided a lot of evidence for the role of the IGF-1R and ligands in the regulation of MMP-2, MMP-9, and u-PA expression in various malignancies including lung cancer [9,16,30]. Furthermore, some studies have reported that the increased invasion and gelatinase activity in cancer cells are associated with the increased activation of PI3-K/Akt [9,17,29,30]. As expected, our results showed that knockdown of IGF-1R, using siIGF-1R1, significantly impaired invasion and migration of A549 cells in the transwell invasion assay and migration assay, and down-regulated the expression of MMP-2, MMP-9, and u-PA at the transcriptional and post-transcriptional levels. Further examination of the levels of total and phospho-Akt demonstrated that the down-regulation of IGF-1R by siIGF-1R1 greatly reduced phospho-Akt levels, but had no obvious effect on total Akt proteins in A549 cells. These data suggested that the down-regulation of IGF-1R might cause IGF-1R-mediated signaling pathway suppression and invasion inhibition of lung cancer cells.

The final important step in metastasis is the establishment of a metastatic colony. In our study, we used tail vein injection of siRNA, which perhaps merits more clinical implication than intratumoral injection of siRNA, to prevent metastasis of the lung cancer cell line A549 [25]. Although the effect of siRNA in human cells is only transitory, we showed in this study that the incidence of micrometastases in the lungs of mice injected with A549-siIGF-1R1 cells was significantly reduced. Similar to this result, remarkable inhibition of IGF-1R in the lungs of tumor bearing mice treated with siIGF-1R1 was observed. Probable explanation is that invasion-related genes were inhibited by siIGF-1R and tumor cells transfected with psiIGF-1R could not adhere to, or colonize in, lung tissues

[25]. The xenograft nude mice experiment provides strong evidence that blocking IGF-1R expression by siRNA significantly impairs invasion and metastasis of lung cancer cells.

The fact that siIGF-1R1 was more effective in decreasing the expression of invasion-related genes and specific downstream genes, and inhibiting the formation of lung metastases in nude mice, compared with siIGF-1R2, is consistent with the results that siIGF-1R1 was more effective than siIGF-1R2 in knocking down the expression of IGF-1R. Therefore, the enhanced IGF-1R silencing resulted in a decrease not only in the IGF-1R expression level but also in the functional level.

In conclusion, these findings confirmed the prediction that IGF-1R silencing by siRNA powerfully inhibits lung cancer cell invasion and metastasis. The results raised the possibility of using IGF-1R silence by siRNA in the future clinical treatment of lung cancer to decrease the high mortality rate, a result of aggressive tumor invasion and metastasis.

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